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1 **Pregnancy to Postpartum Transition of Serum Metabolites in Women with Gestational**
2 **Diabetes**

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11 **Abbreviated title:** Metabolic profiles of postpartum transition

12

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16

17

18 **Abstract**

19 **Context:** Gestational diabetes is commonly linked to development of type 2 diabetes mellitus
20 (T2DM). There is a need to characterize metabolic changes associated with gestational
21 diabetes in order to find novel biomarkers for T2DM.

22 **Objective:** To find potential pathophysiological mechanisms and markers for progression
23 from gestational diabetes mellitus to T2DM by studying the metabolic transition from
24 pregnancy to postpartum.

25 **Design:** The metabolic transition profile from pregnancy to postpartum was characterized in
26 56 women by mass spectrometry–based metabolomics; 11 women had gestational diabetes
27 mellitus, 24 had normal glucose tolerance, and 21 were normoglycaemic but at increased risk
28 for gestational diabetes mellitus. Fasting serum samples collected during trimester 3
29 (gestational week 32 ± 0.6) and postpartum (10.5 ± 0.4 months) were compared in diagnosis-
30 specific multivariate models (orthogonal partial least squares analysis). Clinical
31 measurements (e.g., insulin, glucose, lipid levels) were compared and models of insulin
32 sensitivity and resistance were calculated for the same time period.

33 **Results:** Women with gestational diabetes had significantly increased postpartum levels of
34 the branched-chain amino acids (BCAAs) leucine, isoleucine, and valine, and their circulating
35 lipids did not return to normal levels after pregnancy. The increase in BCAAs occurred
36 postpartum since the BCAAs did not differ during pregnancy, as compared to normoglycemic
37 women.

38 **Conclusions:** Postpartum levels of specific BCAAs, notably valine, are related to gestational
39 diabetes during pregnancy.

40 **Keywords:** Gestational diabetes mellitus, type 2 diabetes mellitus, metabolomics,
41 multivariate statistics, branched-chain amino acids, insulin resistance

42 **1. Introduction**

43 Pregnancy is characterized by extensive metabolic alterations in carbohydrate, fat and protein
44 metabolism to ensure adequate fetal growth and to meet the increased physiological demands
45 of pregnancy, including the additional energy stores required for labor and lactation.

46 Maternal glycemic control depends on the balance between pancreatic β -cell secretion of
47 insulin, insulin clearance, and insulin action in liver, muscle and adipose tissue [1, 2]. Insulin
48 sensitivity changes considerably during pregnancy and declines progressively in late gestation
49 [1, 3]. The fetoplacental unit has been implicated as a major source of maternal insulin
50 resistance, which is rapidly reversed upon delivery [4]. Inadequate β -cell responsiveness add
51 to the increased insulin resistance and leads to gestational diabetes mellitus which is
52 associated with risk of type 2 diabetes mellitus (T2DM) [3, 5].

53 Several risk factors correlate highly with gestational diabetes, including advanced maternal
54 age, fetal macrosomia in a previous pregnancy, obesity, and a family history of diabetes [6].
55 However, early pregnancy screening to identify women at risk for gestational diabetes [2, 7]
56 or postpartum T2DM [3, 5] has not been successful.

57 Metabolomics studies—comprehensive analysis of low-molecular-weight metabolites—have
58 shown great promise in identifying novel pathways and early biomarkers of insulin resistance
59 and T2DM [8, 9]. Several putative metabolic markers and pathways associated with insulin
60 resistance and T2DM have been identified and validated, such as increased levels of
61 branched-chain amino acids (BCAA) and related metabolites [10, 11]. Only a few studies
62 have examined the metabolomics of hyperglycemia or gestational diabetes during pregnancy;
63 however, the findings suggest that T2DM and gestational diabetes share similar features and
64 that their metabolic signatures might partly overlap [12-15]. Thus, metabolomics may be

65 useful for identifying biomarkers and understanding the mechanistic underpinnings of
66 gestational diabetes and increased risk for postpartum T2DM.

67 No study has to our knowledge investigated the unique metabolic transition from a pregnant
68 to a postpartum state and how it differs in women with normal glucose tolerance, women with
69 risk factors for gestational diabetes who remain normoglycemic during pregnancy and women
70 diagnosed with gestational diabetes. We hypothesized that women with gestational diabetes
71 have a unique metabolic profile during the metabolic transition after pregnancy that might
72 help explain pathophysiological mechanisms and potential biomarkers for their elevated risk
73 of postpartum T2DM.

74

75 **2. Material and Methods**

76 **2.1. Sample Collection**

77 To study the postpartum metabolic transition, we included subjects that were sampled both
78 during their third trimester (gestational week 32 ± 4) and postpartum (11 ± 3 months
79 postpartum). Eleven women had gestational diabetes mellitus (GDM group), 24 had normal
80 glucose tolerance (NGT group) and 21 were normoglycemic but at increased risk of GDM
81 (NGT risk group) (Figure 1). The distribution of risk factors for GDM in the three groups is
82 shown in Table 1.

83 For the GDM group, we recruited pregnant women diagnosed with GDM at Sahlgrenska
84 University Hospital, Gothenburg, Sweden according to the 1991 criteria of the European
85 Association for the Study of Diabetes [16]: oral glucose tolerance test 2-hour plasma glucose
86 ≥ 10.0 mmol/l. Capillary blood was analyzed with a HemoCue Glucose+ Analyzer (HemoCue,
87 momoCue, Sweden), and blood glucose concentrations were converted to equivalent plasma
88 glucose concentrations [17]. These women were diagnosed at gestational week 26 ± 6 with an
89 oral glucose tolerance test that showed 2-hour plasma glucose 10.9 ± 0.7 mmol/l. After
90 diagnose they were treated to reach normoglycaemia.

91 Women in the NGT-risk group were recruited at primary health care maternity clinics in the
92 Pirkanmaa region, Finland [18]. Eligible women had at least one of the following risk factors
93 at 8–12 weeks' gestation: body mass index (BMI) ≥ 25 kg/m², GDM or any signs of impaired
94 glucose tolerance or a macrosomic newborn (≥ 4500 g) in any earlier pregnancy, type 1 or 2
95 diabetes in first or second-degree relatives, or age ≥ 40 years. Exclusion criteria were an
96 abnormal oral glucose tolerance test at baseline and type 1 or T2DM before pregnancy, use of
97 neuroleptic drugs, and smoking.

98

99 The NGT group consisted of healthy, normoglycemic pregnant women of normal weight from
100 the Gothenburg area, recruited through advertising at the local maternity wards.

101 All women underwent clinical evaluations during their third trimester (gestational week $32 \pm$
102 4) and postpartum (11 ± 3 months). Fasting blood samples were collected at each visit and
103 analyzed for glucose, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein
104 (LDL), insulin, and free fatty acids (FFA). Samples from the GDM and NGT groups were
105 analyzed at the accredited Clinical Chemistry Laboratory, Sahlgrenska University Hospital
106 (SWEDAC ISO 15189). Samples from the NGT-risk group were analyzed at the UKK
107 Institute for Health Promotion Research, Tampere (glucose, cholesterol, HDL) or the MCA
108 Research Laboratory, Turku, Finland (LDL, insulin, FFA). An aliquot of EDTA plasma from
109 all samples was frozen and stored at -80° C for metabolomics analysis. For analysis of insulin
110 resistance, fasting insulin, glucose, and FFA levels were used to calculate the homeostatic
111 model assessment (HOMA) [19] and insulin sensitivity, revised quantitative insulin
112 sensitivity check index (revised QUICKI) [20].

113 All participants received oral and written information on the study and gave written consent to
114 participate. The studies were approved by the Regional Ethical Review Board, University of
115 Gothenburg. (Dnr 402-08) and of Pirkanmaa Hospital District (Reference number R06230,
116 19.1.2007).
117

118 **2.2. Sample Preparation and Metabolomics Analysis**

119 The run order and sample preparation were designed to minimize biases from sample
120 collection site, sample preparation, and analysis that could confound the interpretation of the
121 results. Samples from the same participant were prepared and analysed in close connection
122 whilst keeping the internal sample order randomized. In total, 112 samples and 33 quality-
123 control samples were analyzed by gas chromatography–time-of-flight mass spectrometry

124 (GC-TOF/MS). Quality control samples, pooled from all included samples, were continuously
125 analyzed. Before GC-TOF/MS analysis, serum metabolites were extracted with MeOH-H₂O
126 by a two-step derivatization procedure [21]. The samples were then injected into an Agilent
127 6890 gas chromatograph equipped with a 10-m fused silica capillary column (inner diameter,
128 0.18 mm) with a chemically bonded 0.18- μ m DB 5-MS stationary phase (J&W Scientific,
129 Folsom, CA). The column effluent was introduced into the ion source of a Pegasus III
130 TOF/MS, GC-TOF/MS (Leco, St Joseph, MI). Drift removal and data normalization are
131 described in the supplemental data.

132 **2.3. Data Processing**

133 To quantify and identify metabolites, we used an in-house MATLAB script. Putative
134 metabolites were extracted by using unique mass channels and retention indices matched to
135 our in-house mass spectral library at the Swedish Metabolomics Centre
136 (www.swedishmetabolomicscentre.se). The data set was filtered to remove double peaks and
137 noisy spectra, and only unique spectral profiles with a relative standard deviation (RSD)
138 <40%, calculated from quality control samples, were included in sample comparison
139 modeling. Criteria set by the Human Metabolome Database (www.HMDB.ca) were used to
140 assign extracted components to different compound classes (amino acids and derivatives,
141 BCAA, carbohydrates, lipids, or no class).

142 **2.4. Statistical Analysis**

143 Groupings, outliers, and trends were detected by principal components analysis (PCA). For
144 each subject, the postpartum sample was subtracted from sample collected during pregnancy;
145 missing data were excluded. Next, a variant of orthogonal partial least squares (OPLS)
146 (OPLS) [22], OPLS-effect projections [23], was used to extract relevant metabolic profiles of
147 the pregnancy to postpartum transition, based on paired analyses of the individual effects (i.e.,
148 the effect of the postpartum transition). Since each subject served as her own control, this

149 strategy minimizes the influence of instrumental drift, site differences, and interindividual
150 variation [23].

151 To validate the multivariate models, P values for the differences between the predefined
152 classes were calculated by analysis of variance (ANOVA) based on the cross-validated OPLS
153 scores (CV-ANOVA); $P < 0.05$ was considered significant. Special consideration was taken
154 to ensure proper cross-validation groups (i.e., that the same participant/replicate was kept in
155 the same group) to reduce the risk of creating overfitted models. A metabolite was considered
156 to contribute significantly to the metabolite profile if it was significantly altered according to
157 the multivariate confidence interval, based on jack-knifing [24], and a significant univariate
158 P -value, both on a 95% significance level. Univariate P values were calculated with the t test
159 (sample size >20) or the Wilcoxon signed-rank test (sample size <20).

160

161

162

163

164 **3. Results**

165 **3.1. Clinical Measurements**

166 Clinical characteristics and measurements in the three cohorts during and after pregnancy are
167 shown in Table 2. BMI during pregnancy was higher in the NGT-risk and GDM groups than
168 in the NGT group ($P < 0.01$). Gestational weight gain was lowest in the GDM group ($P <$
169 0.05 versus NGT-risk). Insulin resistance (HOMA-IR) was higher in the NGT-risk group than
170 in the NGT group ($P < 0.01$), and insulin sensitivity (revised QUICKI) was lowest in the
171 GDM group ($P < 0.05$ vs NGT). Postpartum, the NGT-risk and GDM groups still had
172 significantly higher BMIs and higher waist-to-height ratios than the NGT group.

173 The postpartum shift in clinical measurements is shown in Figure 2. In the NGT group,
174 postpartum plasma glucose and revised QUICKI increased significantly, and HOMA and
175 insulin levels decreased, indicating normalization of metabolic status (Figure 2). The NGT-
176 risk group also increased their postpartum plasma glucose, HOMA, and insulin, indicating
177 normalization of blood glucose, but their insulin sensitivity decreased along with cholesterol.
178 In the GDM group, postpartum glucose and revised QUICKI increased, indicating
179 normalization of insulin sensitivity, but blood cholesterol and LDL were not lowered to the
180 same extent as in the NGT group.

181 The use of dietary supplements in the different groups is found in table S2. It shows no
182 differences in use of supplements between the different groups during pregnancy or
183 postpartum.

184 **3.2. Postpartum Plasma Metabolic Profiles**

185 Initial inspection of the metabolic profiles by principal component analysis (PCA) did not
186 reveal outliers in samples collected during pregnancy or postpartum. The largest systematic
187 variations were related to diagnosis and sample collection site (i.e., the NGT -risk group was
188 separated from the GDM and NGT groups in the first PC) (Figure S1). Since the sampling

189 was longitudinal, we focused on the postpartum metabolic transition profiles for the different
190 diagnosis groups to circumvent differences in site from confounding of interpretation of the
191 results. The postpartum metabolic profile of 66 identified putative metabolites is shown for
192 NGT-risk and GDM groups in Supplemental Table S1.

193 The postpartum metabolic transition models (OPLS-EP) were based on the difference
194 between the pregnancy and postpartum values for each subject. Diagnosis-specific OPLS
195 models (CV-ANOVA $P > 0.001$), which describe the metabolic profile of a postpartum
196 transition, were significantly different for the NGT-risk and GDM groups (Figure 3) but not
197 for the NGT group. Therefore, all findings related to the NGT group are from univariate
198 analysis of single putative metabolites (Table S1). The predictability of the OPLS models
199 (i.e., the percent of the total variation predicted by the calculated latent variable/OPLS
200 component, Q2) was $>75\%$, and two significant components, one predictive and one
201 orthogonal, were extracted for each model. Only the predictive component (the systematic
202 variation related to the postpartum transition) is shown in Figure 2.

203 The postpartum metabolic transition profiles differed in the GDM and NGT-risk groups. In
204 the GDM group, the BCAAs, tryptophan, ornithine, proline, lactose, and a number of hexoses
205 increased significantly postpartum, while glutamic acid and cholesterol decreased
206 significantly. Notably, among the BCAAs valine levels differed most between the study
207 groups and also showed the most pronounced difference between samples collected during
208 pregnancy and postpartum (Figure 4). Indeed, BCAAs did not differ between the NGT and
209 GDM groups (collected at the same site) during pregnancy ($P > 0.92$), but all BCAAs differed
210 significantly ($P < 0.02$) postpartum (Figure 4).

211 Postpartum, asymmetric dimethylarginine and citrulline levels increased significantly in the
212 GDM and NGT-risk groups but not in the NGT group ($P > 0.27$), and the level of
213 polyunsaturated docosahexaenoic acid (DHA, 22:6n-3) decreased significantly in the GDM
214 and NGT groups but not in the NGT-risk group. Also significantly reduced ($P < 0.03$) in the

215 NGT group were postpartum levels of palmitic acid (16:0) and three unsaturated 18C fatty
216 acids, namely linoleic acid (18:2), elaidic acid (18:1, trans), and oleic acid (18:1, cis). In all
217 women, threonine and allothreonine levels decreased and the ketoleucine level increased
218 postpartum. The postpartum transition of all putative metabolites is shown in Table S1.
219

220 **4. Discussion**

221 This study shows that women with GDM have a substantially different metabolic profile
222 during the pregnancy to postpartum transition than women with NGT, including those at
223 increased risk for GDM. Postpartum, the GDM group had a significant increase in the BCAAs
224 (leucine, isoleucine, and valine) and a less pronounced normalization of circulating lipids. The
225 increase was related to higher postpartum BCAA levels in the GDM group, since BCAAs did
226 not differ between the GDM and the NGT group during pregnancy, in line with earlier studies
227 [25, 26]. Postprandial BCAAs 6 weeks postpartum are also higher in insulin-treated women
228 with GDM women than in NGT women [27]. These alterations in protein and lipid
229 metabolism may point to pathophysiological mechanisms and potential biomarkers to predict
230 the development of T2D, after GDM.

231 Pregnancy entails an increased demand for energy, including amino acids, to enable the fetus
232 and placenta to grow. Thus, normal pregnancy induces hypoaminoacidemia, which reduces
233 BCAAs in the circulation, potentially to conserve nitrogen and increase protein synthesis
234 aimed at conservation and accretion of nitrogen by the woman and the fetus [28]. This can
235 explain the conflicting reports on BCAA levels during pregnancy [25, 26, 29]. Lindsay et al
236 showed a decrease in two BCAAs during normal pregnancy, i.e. leucine and valine,
237 suggesting that the amino acids should increase postnatally although no study before have
238 investigated this transition [30]. We could not detect a significant postpartum increase in any
239 of the BCAAs, in NGT or NGT risk groups. However we found a non-significant increase in
240 BCAAs in the NGT group (data not shown). This might indicate that this study was too small
241 to detect the increment back to prepregnancy levels postpartum. Another possibility is that
242 postpartum normalization of BCAA among NGT individuals requires more time than 6-12
243 months.

244 In women with postpartum GDM, increased levels of BCAAs, or other mitotoxic/lipotoxic
245 metabolites from these amino acids, might increase risk for T2DM through their negative
246 effects on β -cell function [31]. Insulin resistance can also be influenced by BCAA
247 metabolites. 3-hydroxyisobutyrate (3-HIB), a catabolic intermediate of the BCAA valine,
248 secreted from muscle cells, activates endothelial fatty acid transport, stimulates muscle fatty
249 acid uptake in vivo, and promotes lipid accumulation in muscle, leading to insulin resistance
250 [32]. 3-Hydroxyisobutyrate levels were higher in muscle from both *db/db* mice and humans
251 with diabetes than in those without. The elevated valine levels in the GDM group can thus
252 contribute to decreased insulin signaling and worsen insulin resistance. However, we could
253 not find any significant postpartum alteration in 3-hydroxyisobutyrate (Table S1).

254

255 We also found significant postpartum alterations in several other interesting amino acids. For
256 example, alanine and arginine levels were increased postpartum in both the NGT-risk and the
257 GDM groups, while leucine and proline were increased only in the GDM group. These amino
258 acids stimulate insulin secretion and could thereby contribute to exhaustion of β -cells by
259 causing endoplasmic reticulum stress [10, 33-35]. We also found increased levels of citrulline
260 in the GDM and NGT-risk groups and of ornithine in the GDM group. Citrulline and
261 ornithine concentrations increase in mice with diet-induced obesity associated with
262 hyperglycemia, hyperinsulinemia, and nonalcoholic fatty liver disease [36]. Chronic elevation
263 of these potential β -cell secretagogues might lead to loss of insulin secretion if inherited
264 abnormalities of beta cell function or mass predispose to the development of diabetes.

265 Insulin sensitivity (revised QUICKI) increased in absolute terms in both the NGT and GDM
266 groups postpartum and was significantly lower in the GDM group and decreased in the NGT-
267 risk group. Concomitantly, insulin resistance (HOMA-IR) decreased postpartum in the NGT
268 and the GDM groups but increased in the NGT-risk group. Notably, the revised QUICKI
269 includes free fatty acid in modeling insulin sensitivity, resulting in a better correlation with

270 the clamp-based index of insulin sensitivity and greater discriminatory power in cases of mild
271 insulin resistance [37, 38].

272 The lack of a significant postpartum metabolic transition profile in the NGT group suggests
273 that the metabolic shift is less pronounced in this group. Nevertheless certain lipid species
274 decreased postpartum, suggesting normalization of lipid levels. Specifically, cholesterol,
275 LDL, HDL, palmitic acid, three unsaturated 18C fatty acids, and DHA decreased. Similarly,
276 in the NGT-risk group, cholesterol, LDL, and HDL decreased during the postpartum
277 transition, but the fatty acids remained unchanged. In the GDM group, however, only
278 postpartum cholesterol and DHA levels decreased. In line with this, we found several
279 circulating lipids (LDL, HDL, cholesterol) that were significantly higher in women with
280 GDM as compared to those with normal glucose tolerance during pregnancy. Elevated
281 circulating lipids during late pregnancy, partly due to rising blood levels of lipolytic placental
282 hormones, may be key for the increase in insulin resistance [39]. Chronic exposure of islets to
283 elevated concentrations of fatty acids can also impair glucose-stimulated insulin secretion [40,
284 41].

285 A large body of evidence implicates lipids, BCAA and other amino acids in the development
286 of tissue disorders, metabolic disease and insulin resistance. These findings suggest that these
287 abnormalities are driven by the combined effects of lipids and BCAA or other amino acids. In
288 addition, there might be interactions of excess BCAAs and lipids in the development of β -cell
289 impairment. The metabolic basis for gradual dysregulation of glucose-stimulated insulin
290 secretion in T2DM is not completely understood, in part because both lipids and amino acids
291 have complex and similar effects on β -cells. Fatty acids can serve as amino acids or
292 secretagogues and increase insulin secretion through a combination of messengers produced
293 during metabolism and through activation of cell-surface G protein-coupled receptors [42,
294 43]. In this way, chronic exposure of islets to elevated concentrations of fatty acids impairs

295 glucose-stimulated insulin secretion. The chain length, degree of unsaturation, and the spatial
296 configuration of fatty acids influence their effects on β -cell function [44].

297 A limitation of this study is the size of the GDM group. Importantly, this was considered in
298 the multivariate analysis, in which each woman served as her own control during extraction of
299 metabolic profiles. This strategy potentially increases statistical power by reducing site- and
300 intra-individual biases that could confound the interpretation of the results. Also, nutritional
301 and physical activity patterns are important factors that might influence the metabolic pattern
302 and should be taken in consideration in future studies.

303 In conclusion, our findings, especially the validity of BCAAs and lipids as potential
304 pathophysiological factors explaining the development to T2DM, negatively affecting β -cell
305 function and insulin sensitivity, need to be further validated in combination with clinical
306 follow-up data on the actual development of T2DM. The ultimate goal is to develop clinical
307 easy-to-use, widely applicable markers to prevent T2DM after GDM in at-risk-women.

308 **6. Acknowledgements and contribution statement**

309 E.C performed the metabolomics analysis and the multivariate statistics, wrote the manuscript
310 and is the guarantor of this work. U.A.H analyzed clinical data and contributed to writing of
311 manuscript, C.G collected and compiled clinical data. K.B reviewed and edited the
312 manuscript and contributed to discussion. J.P compiled clinical data R.L collected clinical
313 samples and contributed to the discussion. T.O wrote the manuscript and contributed to
314 discussion. A.H designed the study, wrote the manuscript, collected clinical samples and is
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322

323

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437

438 **Figure Legends**

439 **Figure 1 – Study protocol.** Flow chart illustrating included women that were either
440 normoglycemic (NGT), normoglycemic with increased risk for developing gestational
441 diabetes (NGT risk) or diagnosed with gestational diabetes (GDM) and time table for
442 sampling.

443 **Figure 2—Postpartum shift in clinical measurements.** Absolute changes in concentration
444 from the third trimester (gestational week 32 ± 0.6) to postpartum (10.5 ± 0.4 months) for
445 clinical measurements and mathematical models of insulin resistance (HOMA-IR) and insulin
446 sensitivity (revised QUICKI). Values are mean \pm SD. * $P < 0.05$ postpartum versus late
447 pregnancy (paired t test. # $P < 0.05$ (one-way ANOVA and Tukey posthoc test).

448 **Figure 3—Multivariate analysis.** Diagnosis-specific OPLS models displaying the metabolic
449 profile of the postpartum transition (OPLS model weights, $w^*[1]$), i.e. the significantly altered
450 plasma metabolites when comparing samples collected during the third trimester (gestational
451 week 32 ± 0.6) to those collected postpartum (10.5 ± 0.4 months). (A) NGT -risk group. (B)
452 GDM group. No significant model was obtained for the NGT group. Plasma components with
453 positive axis values were higher postpartum and those with a negative axis values were lower
454 than during pregnancy. Only components that were altered significantly postpartum are
455 shown (significant by the OPLS multivariate 95% confidence interval (based on jack-knifing)
456 and univariate $P < 0.05$ (paired t test).

457 **Figure 4—Branched-Chain Amino Acids.** Relative concentrations of the branched-chain
458 amino acids (BCAA, valine, leucine and isoleucine), detected by GC/MS- based
459 metabolomics. All BCAAs were higher postpartum (black dots) in the gestational diabetes
460 mellitus (GDM) group, $P < 0.01$) than during pregnancy (white dots). No significant
461 postpartum alterations were detected in the normal glucose tolerance (NGT) or NGT-risk
462 groups. All three BCAAs differed between NGT and GDM group postpartum ($P < 0.02$) and

463 valine and isoleucine differed between GDM and NGT-risk postpartum; no difference were
464 seen during pregnancy. The unique mass channel for each amino acid used for quantification
465 is stated on each y-axis. Red line indicates mean values and the grey box represent 95%
466 standard deviation of the sampling distribution.